AAPS Pharmsci 2001; 3 (1) article 7 (http://www.pharmsci.org/)

Lipid-Based Delivery of Combinations of Antisense Oligodeoxynucleotides for the *In Vitro* Inhibition of HIV-1 Replication

Submitted: August 14, 2000; Accepted: January 30, 2001; Published: February 12, 2001.

Carole Lavigne,* Jocelyn Yelle, Gilles Sauvé,
Département de Microbiologie et Immunologie, Faculté de Médecine, Université de Montréal, Montréal, Québec, Canada H3C 3J7
Institut Armand-Frappier, Université du Québec, Laval, Québec, Canada H7N 4Z3

Alain R. Thierry
Laboratory of Tumor Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892-4255, USA
MedinCell Project, 31240 L'union, France

*New Address: Institut de recherche medicale Beausejour, Centre d'Oncologie Dr Léon Richard, Moncton, Nouveau-Brunswick, Canada, EIC 2Z3

ABSTRACT We evaluated a new approach to AIDS therapy by using combinations of oligodeoxynucleotides (ODNs), delivered with a lipid-based carrier system, that target different HIV viral genome sites. We identified some of the factors that seem to influence the effectiveness of a combination strategy in cell cultures including ODN concentrations, type of infection (acute vs chronic), backbone modification of the ODN, and the number of sequences. When delivered by the DLS carrier system, some advantages of using a combination of ODNs over treatment with only one ODN could be observed in acute infection assays but not in the chronic infection model. These results suggest that in the acute infection model, the 3 different antisense ODNs in the "cocktail" might block an early step of virus replication by combined inhibitory effects. Various combinations of phosphorothioate-modified (PS) and unmodified oligonucleotides delivered by the DLS system were compared for their antiviral activity in a long-term acute assay using HIV-1 (IIIB strain)-infected MOLT-3 cells. The most effective combination had 3 phosphorothioate antisense ODNs: Srev, SDIS, and SPac (>99% inhibition at 100 pM). However, the additive effect determined when using ODN combinations was rather low, revealing the high level of nonsequence specificity in HIV-1 cell culture models. Data illustrated the high sequence nonspecific activity of ODNs, especially when comparing activity of antisense ODNs with activity of random control sequence ODNs. The latter exhibited an inhibitory effect similar to that of antisense ODNs under our experimental conditions. Nevertheless, we demonstrated that it is possible to achieve high anti-HIV activity by using, in combination, picomolar range concentrations of antisense oligonucleotides complexed to a lipid-based carrier system such as the DLS system, without increasing cell toxicity.

KeyWords: antisense, oligonucleotides, HIV, drug delivery

INTRODUCTION

Antisense oligonucleotides are new antiviral agents for HIV infection that have shown potential therapeutic application against HIV-1 (1-3). DNA oligonucleotides hybridize to target RNA by Watson-Crick base pairing to inhibit translation by simply blocking ribosomal reading or inducing degradation of the RNA part of duplexed DNA/RNAs by activation of RNase H (4). DNA with natural phosphodiester linkages has been found to be rapidly degraded by nucleases and to poorly cross cellular membranes. To overcome these problems, a first generation of chemically modified oligonucleotides has been developed based on backbone modifications. Phosphorothioate-modified (PS) oligonucleotides, in which an oxygen atom of the phosphodiester linkage has been replaced by a sulfur atom, are the most common oligonucleotide analogs to have been investigated. Several regions of the HIV genome have been targeted by phosphorothioate antisense oligonucleotides including *rev*, *tat*, *gag*, *pol*, *env*, and noncoding regions such as the 5'-LTR. Antisense oligonucleotides were effective in both acute and chronic infections (5-8).

Recently, 2 novel antisense sequences named SDIS and SPac, which are derived from the 5'-end of the HIV-1 genome, have been developed in our laboratory and tested in acutely and chronically infected cells against laboratory and clinical HIV-1 isolates. The 26-mer phosphorothioate antisense molecule SDIS is complementary to a highly conserved sequence localized between the primer binding site and the major splice donor site spanning nucleotides +245 to +270 (9). This sequence is considered to be essential for HIV-1 RNA dimerization (10, 11) and encapsidation (12, 13). The 30-mer phosphorothioate antisense molecule SPac is

Corresponding author: Alain R. Thierry, PhD. MedinCell Project, 6, rue des Monts du Vivarais, 31240; L'union, France. E-mail: thierry1@micronet.fr

complementary to a sequence localized between the major splice donor site and the first ATG *gag* initiation codon (+295 to +324 nt) and corresponds to the packaging signal *psi* (ψ) (13-15). Results of the anti-HIV assays done with SDIS and SPac were encouraging because the 2 sequences were found to be as potent as the well-documented antisense oligonucleotides anti-*rev* (5) and GEM 91 (16) in inhibiting HIV-1 replication in vitro. GEM 91 is a 25-mer oligodeoxynucleotide directed against the translation initiation site of HIV-1 *gag* mRNA, which has been extensively studied for its anti-HIV activity (3, 7, 17).

The study evaluated a new approach for the treatment of HIV infection by using a combination of different oligodeoxynucleotides (ODNs) complexed to a lipid-based carrier system. Our intention in using such a combination strategy was to interfere simultaneously at different levels in the replication of HIV-1 by using different ODNs that have distinct targets on the viral genome in order to improve the efficacy of oligonucleotide technology. The potential advantages of a combination approach using antisense technology are multiple and include the presence of additive inhibitory effects on HIV-1 replication by a combination of different mechanisms of action; the possibility of targeting different viral genome sites at the same time, thereby minimizing the emergence of escape mutants; and the reduction of individual oligonucleotide concentration, making clinical application more feasible.

Table 1. Sequence of the Oligonucleotides Used in Combination for the Treatment of HIV-1 Infection In Vitro

ODNs and Cocktails	Nucleotide Sequence and Cocktail Composition
Srev or rev (antisense)	5'-TCGTCGCTGTCTCCGCTTCTTCCTGCCA-3'
SDIS (antisense)	5'-CTCTTGCCGTGCGCGCTTCAGCAAGC-3'
DIS (antisense)	5'-CTCTTGCCGTGCGCGCTTCAGCAAGCCG-3'
SPac (antisense)	5'-TCTAGCCTCCGCTAGTCAAAATTTTTGGCG-3'
RS (random)	mixture of all 4r nucleotides
RD (random)	mixture of all 4 nucleotides
Cocktail -1	rev-DIS-RD
Cocktail - 2	rev-DIS-RS
Cocktail - 3	Srev-SDIS-RS
Cocktail - 4	Srev-SDIS-Spac
Cocktail - 5	Srev-SDIS-SPac-RS

Oligonucleotides S*rev*, SDIS, SPac, and RS (random) were synthesized with a phosphorothioate backbone. Oligonucleotides *rev*, DIS, and RD (random) were synthesized with phosphodiester linkage.

We investigated the ability of a combination of ODNs used in HIV-infected cell cultures to provide the advantages expected from a multitargeting approach. We first compared the level of the antiviral activity, the viral breakthrough, and the effects on cell survival of a 3-ODN combination with single-ODN regimens in both acute and chronic infection models at low concentrations by using the DLS lipid-based delivery system. DLS formulation was recently evaluated in our laboratory for in vitro delivery of ODNs and was found to be a successful approach for enhancement of cellular uptake and antisense activity of this class of compounds (18). Second, to improve the potency of the combination approach with oligonucleotide analogs, we compared the anti-HIV activity of different combinations of oligonucleotides delivered by the DLS carrier system in acutely infected MOLT-3 cells. Multiple steps in the HIV replicative cycle have been targeted by using phosphorothioate-modified and unmodified antisense molecules with different RNA targets (translation of the Rev protein, dimerization site, and packaging signal site) and scrambled sequences known to have nonspecific effects on HIV replication. To our knowledge, the anti-HIV activity of a continuous multidrug regimen with unmodified and phosphorothioate-modified ODNs delivered by a lipid-based carrier system in vitro has never been investigated for the treatment of HIV infection.

MATERIALS AND METHODS

Oligodeoxynucleotide sequences

Oligodeoxynucleotides were synthesized by an automated DNA synthesizer (BioServe Biotechnologies, Laurel, MD) with a sulfur atom introduced at each phosphodiester linkage for phosphorothioate-modified ODNs. Synthesis was carried out on a 1 μM scale. The oligonucleotides were deblocked, desalted, and purified by polyacrylamide gel electrophoresis (PAGE). The ODNs were quantitated by UV absorbance at 260 nm (1 OD ≈ 33 μg of DNA). Sequences used in combination regimens are shown in Table 1. Three antisense sequences known to have anti-HIV activity were used: SDIS and SPac, which are phosphorothioate-modified sequences complementary to a non-coding portion of the 5'-end of the HIV-1 genome and the phosphodiester form DIS; and a 28-mer ODN complementary to the 5'-end sequence of HIV-1 *rev* mRNA in either a

phosphorothioate (Srev) or phosphodiester (rev) form (5). SDIS is complementary to a highly conserved sequence considered essential for HIV-1 RNA dimerization and encapsidation. SPac is complementary to the RNA packaging signal *psi* (ψ). Scrambled ODNs that are not complementary to any sequence motifs of the HIV-1 genome were also used in combination regimens: a 28-mer random sequence in a phosphorothioate (RS) or phosphodiester (RD) form. The compositions of the 5 different combinations evaluated herein are shown in Table 1.

Cells and virus

The $CD4^+$ human lymphoid cell line MOLT-3 was kindly provided by Dr R.-P. Sekaly (Clinical Research Institute of Montreal, Québec, Canada) and the $CD4^+$ human lymphoblastoid cells chronically infected with HIV-1 (IIIB) (H9/HTLV-IIIB NIH 1983) (19, 20) were obtained from the NIH AIDS Research and Reference Reagent Program (Rockville, MD). Uninfected and infected cells were cultured in RPMI 1640 culture medium (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum, L-glutamine (4 mM), and gentamycin (50 μg/mL) at 37°C in a 5% CO_2 atmosphere. HIV-1 laboratory strain IIIB was obtained from Advanced BioScience Laboratories Inc (Kensington, MD) and was used to infect MOLT-3 cells.

Preparation of ODN-lipid complexes

DLS liposomes consist of small unilamellar vesicles approximately 50 nm in diameter, which can complex with ODNs in an interactive molecular manner. After addition of DNA or ODN to DLS liposomes, a completely different multilamellar structure is formed, with particle size ranging from 100 to 150 nm, that is very stable and shows great homogeneity. DLS liposomes were formed by mixing 1 mg of dioctadecylamidoglycylspermidine (DOGS; Promega, Madison, WI) and 1 mg of dioleoylphosphatidylethanolamine (DOPE; Sigma, Saint Louis, MO) as previously described (21, 22). Oligonucleotides were first complexed to DLS liposomes separately in sterile deionized water at a final concentration of 0.26 mg/mL. The preparations contained 10 μg of ODNs per 38 μL of rehydrated lipids. The preparations were incubated at room temperature for at least 30 minutes before they were added to the cells. Dilution of DLS-associated ODNs in sterile deionized water was made to obtain appropriate concentrations, and combinations of different ODNs were made at an equal molar ratio after complexation. DLS-ODN complexes were stored at 4°C until the next treatment 3 or 4 days later. Fresh DLS-ODN complexes were prepared every week (every 2 treatments).

Antiviral Assays

To compare the antiviral activity of the 3-ODN combination Srev-SDIS-SPac with that of single-ODN regimens at low concentrations, we used MOLT-3 cells acutely infected with HIV-1 laboratory strain IIIB as a model. Cells were infected at a viral titer of $TCID_{50}$ = 2000 (viral stock 2000 $TCID_{50}$/mL). After 2 hours adsorption at 37°C, infected and control cells were washed twice with RPMI culture medium to remove unabsorbed virus and were resuspended in fresh complete medium. Cells were plated into 96-well microtiter plates at a concentration of 4×10^5 cells/mL and were treated for up to 28 days with different DLS-associated ODNs added either alone or in combination. The ODN combinations were formed by adding equal amounts of each DLS-ODN preparation. For example, to obtain a final concentration of 100 pM with a combination of 3 ODNs, each ODN was added to the formulation at a concentration of 33 pM. Every 3 or 4 days, cells were split to 4×10^5 cells/mL and supernatants were collected to determine the presence of HIV.

To test the efficiency of the 3-ODN combination in chronic infection, we used H9 cells chronically infected with HIV-1 (IIIB) as a model. Cells were plated into 96-well microtiter plates at a concentration of 4×10^5 cells/mL, and ODN combinations were added as DLS-ODN preparations at 0.1 and 1 pM final concentrations. The cells were kept in culture for 4 days, and HIV-1 replication was determined by the p24 antigen assay.

To compare the antiviral activity of different combinations of ODNs in a long-term assay, MOLT-3 cells were infected with HIV-1 laboratory strain IIIB at a viral titer of $TCID_{50}$ = 1000 as described above. Cells were plated into 96-well microtiter plates at a concentration of 4×10^5 cells/mL and treated for up to 21 days with different DLS-associated ODN combinations at 100 pM final

concentrations. Every 3 or 4 days, cells were split to 4×10^5 cells/mL and supernatants were collected to determine the HIV-1 activity.

Determination of virus replication

Virus replication was determined by detection of p24 HIV-1 viral core antigen in cell-free supernatants by a p24 antigen-capture assay (Coulter Immunology, Frederick, MD). Cell viability was monitored by the tetrazolium-based colorimetric cell proliferation (Cell viability was monitored by use of a colorimetric assay, based on the reduction of the tetrazolium salt (23)).

Statistical analysis

Experimental groups were expressed as mean ± standard deviation and compared with control groups or different treatment groups using the single-factor analysis of variance. When statistical significance ($P < .05$) was reached with the F test, comparisons of the means were then performed using either the Tukey-HSD test or the Student *t* test. A *P* value of .05 or less was considered significant.

RESULTS

Inhibition of viral production in acutely infected MOLT-3 cells by DLS-complexed ODNs used either alone or in combination.

To evaluate the beneficial effects of the 3-ODN combination regimen on HIV-1 replication at low ODN concentrations, we used the DLS delivery system to achieve subnanomolar concentrations. We compared the antiviral activity of the 3-ODN combination with each of the ODNs used individually in MOLT-3 cells acutely infected with HIV-1 (IIIB) in a long-term assay. The ODN "cocktail" and each individual ODN were complexed to DLS formulations before being added to the culture medium, and viral production in cell culture supernatants was determined every 3 to 4 days after infection. The level of p24 antigen in each well was monitored for 28 days, and the highest values obtained in each sample were compared with each other. Results are presented in Figure 1 as percent inhibition of p24 production compared with infected, untreated cell culture controls. Antisense ODNs Srev and SPac showed limited activity at 10 pM concentration when used individually (inhibition of 28% and 11%, respectively), while SDIS showed a level of inhibition of 39%. At the same concentration, a higher level of inhibition was achieved when the same antisense ODNs at 10 pM final concentration were used in combination (60% inhibition) compared with Srev and SPac alone ($P = .05$). When the dose of ODNs was increased to 100 pM, antiviral activity was improved in cell cultures treated with antisenses used alone: 41% for Srev, 53% for SDIS, and 37% for SPac. However, no significant difference between the inhibitory effects of the combination regimen Srev-SDIS-SPac and the single-ODN regimens could be detected at 100 pM. Random PS ODN and sense SDIS delivered with the DLS system showed a 62% and 53% inhibition at 10 pM and a 68% and 61% inhibition at 100 pM, respectively.

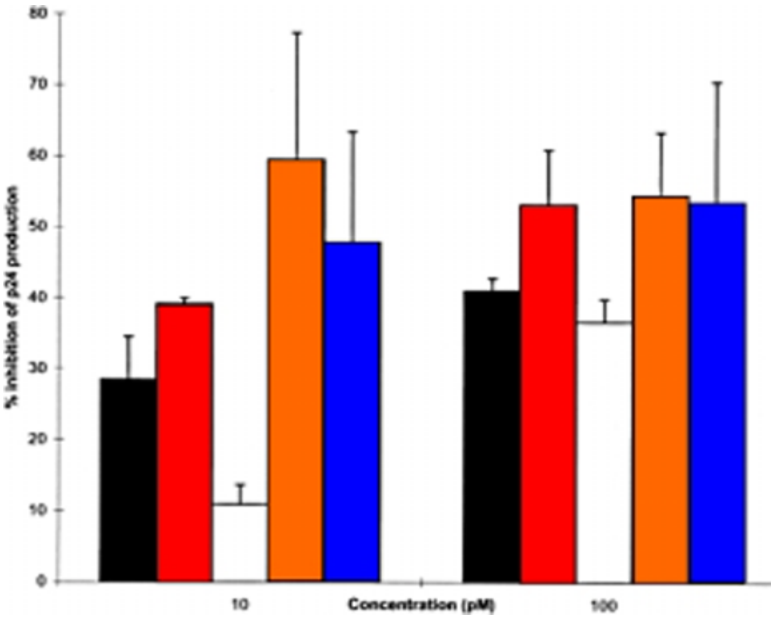

Figure 1. Long-term anti-HIV assay in acutely infected MOLT-3 cells. The cells were infected with HIV-1 (IIIB) and cultured in presence of antisense oligonucleotides added either individually or in combination for 28 days (viral stock 2000 $TCID_{50}$/mL). Each individual ODN and the 3-ODN cocktail (Srev-SDIS-SPac) were delivered by the DLS carrier system at final concentrations of 10 and 100 pM. The highest level of p24 antigen in each assay was determined, and the data are given in terms of percent inhibition of p24 production, compared with the highest level of p24 antigen found in infected, untreated cell cultures. Values represent the mean (± SD) of 2 separate experiments carried out in triplicate.

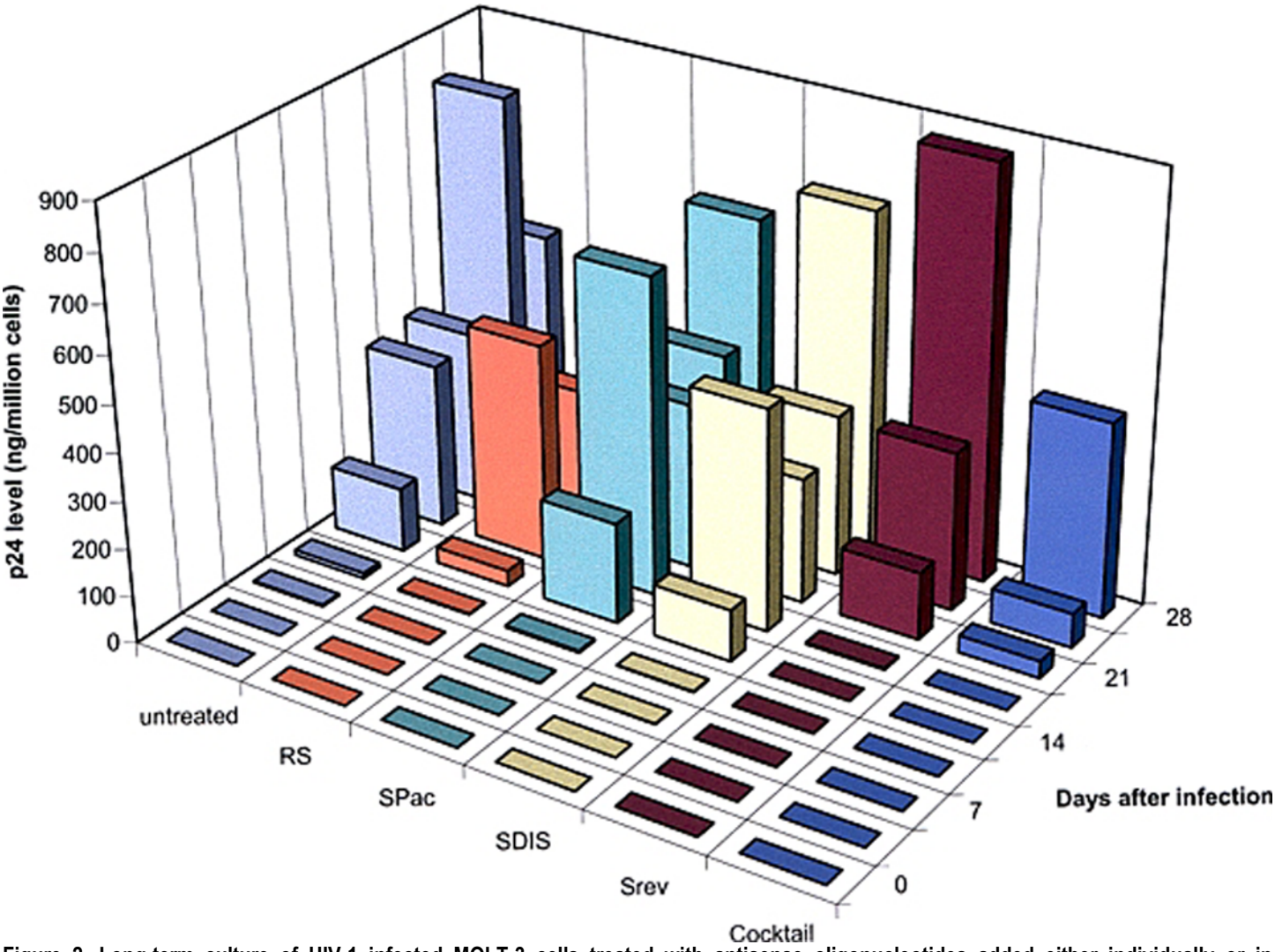

Figure 2. Long-term culture of HIV-1 infected MOLT-3 cells treated with antisense oligonucleotides added either individually or in combination delivered by the DLS carrier system. Cells were infected (viral stock 2000 $TCID_{50}$/mL) and treated with ODNs individually or in combination at 100 pM final concentration. The 3-ODN cocktail was composed of Srev, SDIS, and SPac antisense ODNs at a concentration of 33 pM each. The level of p24 production was determined in the culture supernatants every 3 to 4 days by HIV-1 antigen capture assay. Values represent the mean of duplicate determinations (SD < 15%).

Suppression of viral breakthrough by DLS-complexed ODNs used individually or in combination in acutely infected MOLT-3 cells.

To determine if our 3-ODN combination was able to delay viral emergence for a longer period of time than a single ODN when used at subnanomolar concentrations, we used MOLT-3 cells infected with HIV-1 (IIIB) in a long-term assay. Cells were treated with the individual ODNs or with the 3-ODN combination delivered by the DLS carrier system at 100 pM for 28 days. Results are shown in Figure 2. In infected, untreated cell cultures, HIV-1 was detected 14 days after infection. Neither SPac nor SDIS used individually at 100 pM were able to delay viral breakthrough for longer than 14 days. In contrast, Srev at 100 pM used alone delayed the emergence of virus for up to 21 days. The 3-ODN regimen with 33 pM of each antisense ODN showed the best activity, retarding the emergence of HIV-1 for at least 21 days, with a decreased level of viral replication still evident at 28 days.

Short-term antiviral activity of ODNs used alone or in a 3-ODN combination in chronically infected cells.

We investigated the ability of the 3-drug cocktail to inhibit viral production by chronically infected

H9/HTLV-IIIB cells and compared the antiviral activity with that of single-ODN regimens. Cells were treated for 4 days with ODNs complexed with the DLS formulation added either separately or simultaneously as a 3-ODN combination, and viral replication was determined in cell culture supernatants. Results are shown in Figure 3. At 0.1 pM, a slightly lower antiviral effect was observed with the 3-ODN combination (66% inhibition) compared with SDIS (81%) used individually (P = .05). In contrast, no statistical difference between the cocktail (66%) and Srev (73%) or SPac (77%) used alone was observed at this same concentration. When the dose was increased to 1 pM, no statistically significant differences between antiviral activity of single-ODN and 3-ODN regimens were seen because all regimens were equally efficient in inhibiting viral production (76% to 83% for single-ODN regimens and 80% for the 3-ODN cocktail). Random PS ODN delivered with the DLS system showed a 73% inhibition at 10 pM.

Comparison of the anti-HIV activity of different combinations of oligonucleotides delivered by the DLS system.

To compare the effectiveness of different ODN cocktails, we evaluated their ability to inhibit viral replication in acutely infected MOLT-3 cells in a long-term model treatment. Drugs were delivered by the DLS carrier system, and antiviral activity at the peak of viral production was evaluated in cultures at a final concentration of 100 pM (Figure 4). Data were expressed in terms of percent inhibition of p24 production compared with infected, untreated cell cultures. Results indicate that cocktails containing the random phosphodiester or phosphorothioate-modified ODNs (cocktails 1, 2, 3, and 5) displayed lower activity than did cocktail 4, which was devoid of random ODN. In this assay, the 3-S-ODN cocktails 4 (Srev-SDIS-SPac) and 3 (Srev-SDIS-RS) were more effective than the 4-S-ODN cocktail 5 (Srev-SDIS-SPac-RS) (P < .005 and P < .01, respectively). Cocktail 4 was the most effective, with 92% inhibition of viral replication.

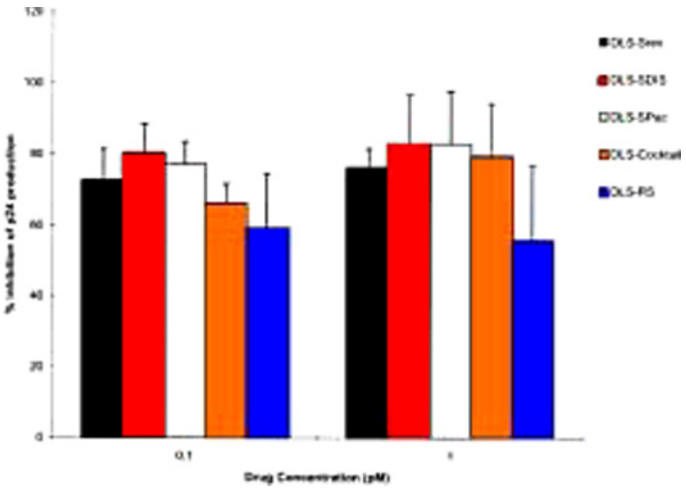

Figure 3. Short-term assay in H9 cells chronically infected with HIV-1 (IIIB) . Cells were cultured in the presence of antisense ODNs delivered by the DLS carrier system, either individually or simultaneously in a 3-drug combination. After 4 days, p24 antigen was determined in cell culture supernatants. Values represent the mean (± SD) of at least 2 separate experiments done in duplicate.

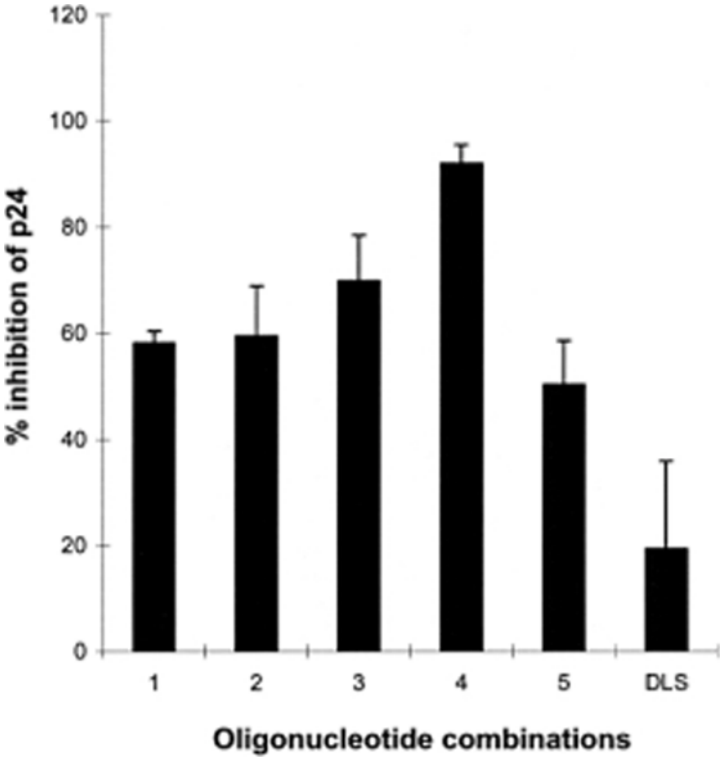

Figure 4. Comparison of the anti-HIV activity of various oligonucleotide combinations in acutely infected MOLT-3 cells (viral stock 1000 $TCID_{50}$/mL). ODN combinations were delivered by the DLS carrier system at 100 pM final concentration. Data are given in terms of percent inhibition of p24 antigen production compared with infected, untreated control cultures at the peak of infection. 1: combination of unmodified antisense rev, DIS, and random sequence RD at 33 pM each; 2: combination of rev, DIS, and phosphorothioate random sequence RS at 33 pM each; 3: combination of phosphorothioate antisense Srev, SDIS, and RS at 33 pM each; 4: combination of Srev, SDIS, and SPac at 33 pM each; 5: combination of Srev, SDIS, SPac, and RS at 25 pM each, while « DLS » represents cells treated with DLS formulation only, at 100 pM. Values represent the mean (± SD) of triplicate experiments.

Differences between cocktail 4 and other cocktails were evident as soon as 3 days after the beginning of the treatment (Figure 5). In fact, cocktail 4 showed high antiviral activity after only 3 days of treatment (>99%) and maintained a high level of inhibition during the entire experiment (21 days). Cocktail 1 (rev-DIS-RD) showed maximum inhibitory effect at 7 days (74%), a level of activity that decreased thereafter. Cocktail 2 (rev-DIS-RS) showed maximum activity at day 14 (88%), after which the antiviral activity decreased steadily. Cocktail 3 (Srev-SDIS-RS) reached its peak of activity only after 17 days (86%), while cocktail 5 (Srev-SDIS-SPac-RS) showed its maximum inhibitory effect 7 days after treatment (58%). Therefore, cocktails 4 and 5, which contained the 3 antisense sequences, reached their maximal antiviral activity very soon after infection and maintained their level throughout the experiment, compared with the other cocktails, in which more variations in anti-HIV activity were observed or in which the maximum inhibitory activity was reached later after infection. Cocktail 5, however, showed lower activity than cocktail 4 throughout the experiment. At day 21, cocktails 3 and 4 were the 2 most potent combinations (72% and 79% inhibition, respectively), while cocktail 2 was the least efficient combination (48% inhibition). However, differences between the antiviral activity of cocktails 3, 4, and 5 were not statistically significant at this time.

Cell survival of cultures acutely infected with HIV treated with the cocktail.

To determine the effects on cell survival of the 3-ODN combination delivered by the DLS system, we compared the viability of infected MOLT-3 cells treated with each ODN used individually with that of infected cultures treated with the ODNs used in combination at 100 pM final concentration. After 28 days of treatment, cell counts were compared (Figure 6). The data are expressed in terms of percent cell viability compared with infected, untreated cell cultures. Overall, similar rates of cell survival were observed with the 3-ODN combination and the single-ODN treatments. The 3-ODN combination with 33 pM Srev, SDIS, and SPac caused less than 15% cell mortality compared with control cultures throughout the 28-day experiment.

Effects on cell survival of the 3-ODN combination treatment, with 33 pM each of Srev, SDIS, and SPac delivered by the DLS carrier system, were also evaluated in chronically infected H9/HTLV-IIIB cells. After 7 days, infected cells treated with the antisense ODNs used either individually or in combination at 100 pM showed no significant loss of viability compared with untreated control cultures (Table 2). Taken together, our results show that when ODNs were used in combination at 33 pM for each antisense ODN, no synergistic toxicity was observed compared with ODNs used individually at 100 pM.

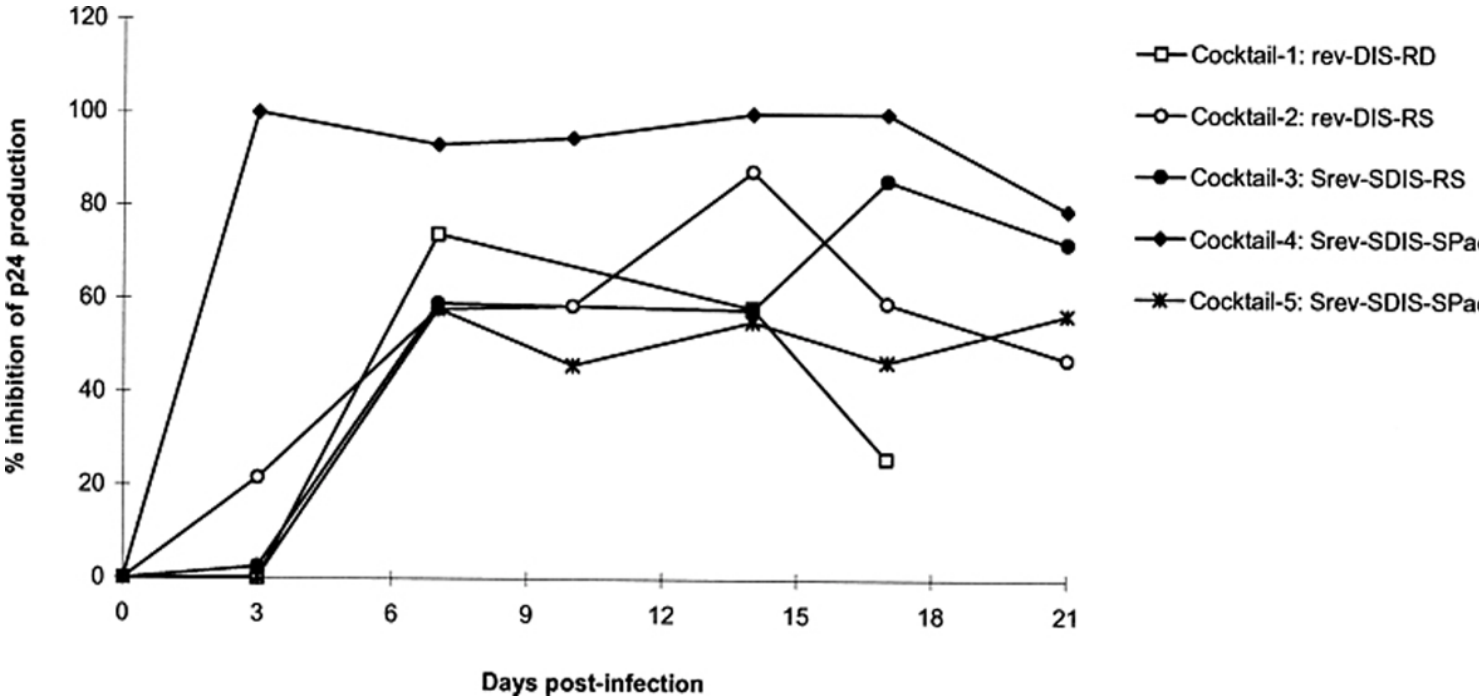

Figure 5. Long-term inhibition of HIV-1 strain IIIB by different oligonucleotide combinations in acutely infected MOLT-3 cells (viral stock 1000 $TCID_{50}$/mL). Cells were treated with various combinations of ODNs delivered by DLS formulation at 100 pM final concentration. ODNs were added in equal molar ratio to form the different combinations. Results are averages (± SD) of 2 separate experiments done in duplicate (SD < 20%).

DISCUSSION

In this report, the in vitro antiviral effects of a combination of 3 phosphorothioate antisense oligonucleotides directed against the HIV-1 genome were evaluated in cell lines at subnanomolar concentrations using the DLS delivery system, which has been shown to be highly efficient in delivering DNA oligonucleotides in vitro (18).

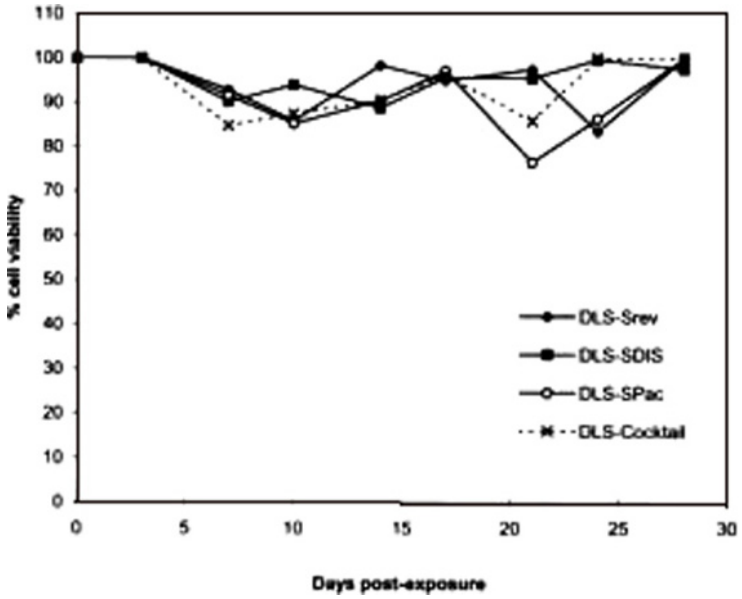

Figure 6. Cell survival of MOLT-3 cell cultures treated with antisense drugs delivered by the DLS carrier system. Cells acutely infected with HIV-1 (IIIB) were exposed to various antisense oligonucleotides added either individually or in a 3-drug combination at 100 pM final concentration for 28 days. Cell counts were determined by the MTT assay every 3 to 4 days. The data are reported in terms of percent of cell viability, compared with infected, untreated cell cultures. Values represent the mean of 2 separate experiments done in duplicate. (SD < 15%).

Table 2. Viability of H9 Cell Cultures Chronically Infected with HIV-1 (IIIB), Ttreated with DLS-associated Antisense Oligonucleotides Used Individually or In Combination at 100 pM.

Treatment	% Cell Viability
DLS-Srev	87 ± 9
DLS-SDIS	95 ± 5
DLS-SPac	91 ± 3
DLS-Cocktail	95 ± 6

Cell counts were determined after 7 days of exposure by the MTT assay.
Percent of cell viability (± SD) was calculated by comparison to chronically infected, untreated cell cultures. The cocktail is a combination of S*rev*, SDIS, and SPac at 33 pM.

Here, MOLT-3 cells acutely infected with HIV-1 (IIIB) were used as an acute infection model and H9 cells chronically infected with HIV-1 (IIIB) as a chronic infection model. First, when antisense ODNs complexed with DLS-liposomes were tested in an acute infection model using MOLT-3 cells infected with HIV-1 (IIIB) in a long-term assay, higher inhibition of p24 production was achieved with the multidrug regimen compared with some single-ODN regimens (1.5 to 5.4-fold at 10 pM and up to 1.5-fold at 100 pM) (Figure 1). Also, in this same model, emergence of virus could be delayed for 7 days in infected cells treated with the 3-ODN combination compared with infected, untreated control cultures (Figure 2). The cocktail prevented the emergence of viral replication more effectively than did SDIS and SPac used individually but was as effective as Srev used alone. However, throughout the experiment, the viral production was lower in cells treated with the 3-ODN combination than in cells treated with ODNs alone or in untreated cells. These results indicate that there are effectively some advantages in using combinations of oligonucleotides at low concentrations in acute infection. These advantages are not associated with an increase of cell toxicity because our assays on cell viability showed that antisense ODNs used in combination did not impair cell survival in this model (Figure 6). Nevertheless, the acute infection cell culture model highlighted the high activity of random ODNs at a level similar to that obtained with antisense sequences. Consequently, we further tested combinations including random modified ODN sequences.

In contrast to acute infections, no beneficial effects were obtained with the multidrug regimen compared with single-drug regimens in our chronic infection model. Nevertheless, in those cells, we were still able to inhibit viral production by more than 75% by using very low concentrations of each ODN included in the combination (0.33 pM) and around 66% when the concentration of each ODN was reduced to 0.033 pM. To our knowledge, anti-HIV activity of antisense oligonucleotides at such low concentrations has never before been reported for delivered ODNs, showing the high potency of the DLS delivery system. Antiviral activity at such low concentrations might eventually prove useful for in vivo application to avoid nonspecific effects and toxicity. Furthermore, our results on cell viability show that in combining antisense ODNs, we did not increase

cytotoxic effects compared with those of ODNs used individually (Table 2).

Taken together, these results suggest that a combination of ODNs might present some advantages in acute infection but not necessarily in chronic infection. In chronically infected cells, the viral DNA is integrated to the cell genome and, thus, the early steps of infection such as virus entry and reverse transcription are not available for ODN activity and only the post-integration events can be targeted by the antisense molecules. Therefore, the advantages of a multitargeting approach seen in our acute infection model might be mainly due to non-antisense and/or antisense additive or synergistic effects displayed during early pre-integration steps such as reverse transcription. Antisense ODNs SDIS and SPac might block reverse transcription by an antisense mechanism if hybridization with viral RNA occurs or by non-antisense mechanisms such as binding to reverse transcriptase (24). Furthermore, because ODNs were complexed to a synthetic carrier, they should not directly interact with events taking place at the cell membrane such as virus adsorption to the host cells and virus entry and/or fusion as reported in a recent study (25), thus eliminating potential non-antisense activity at these levels as an explanation for the higher level of antiviral activity of our cocktail seen in acute infection. In the previous study cited above (25), the ability of phosphorothioate oligonucleotides encapsulated in immunoliposomes to inhibit the cell-to-cell transmission of virus by HIV-induced syncytium formation was evaluated in comparison with oligonucleotides free in solution. When the phosphorothioate oligonucleotides were added free to uninfected $CD4^+$ C8166 cells co-cultured with chronically HIV-1 infected CEM cells expressing gp120 molecules, the formation of syncytia was completely blocked, whereas liposome-encapsulated oligonucleotides failed to block syncytium formation. These results demonstrate that liposomal encapsulation can prevent oligonucleotides from binding to CD4 and to the V3 loop of viral gp120 and, therefore, block extracellular inhibition of virus at the steps of viral entry and fusion with the cell membrane.

To improve our cocktail of oligonucleotides and to better elucidate the mechanisms of activity of the ODN combinations, we evaluated several different combinations of 3 or 4 oligonucleotide sequences, unmodified and phosphorothioate-modified (PS), in MOLT-3 cells acutely infected with HIV-1 (IIIB). The combination containing 3 PS-ODNs was found to be more potent in inhibiting HIV-1 replication than was cocktail 1 composed of 3 unmodified ODNs (Figures 4 and 5), indicating that nonspecific inhibition due to backbone modification might be advantageous in enhancing the antiviral activity of a combination of ODNs. In some cocktails, we included a random sequence either in an unmodified or phosphorothioate-modified form to see if we could improve the efficacy of our cocktails by including a nonsequence-specific activity. The 3-antisense combination Srev-SDIS-SPac showed the highest antiviral activity (Figures 4 and 5) and was the only cocktail to show a high level of inhibition as soon as 3 days after infection and to maintain its high level of activity throughout the experiment (Figure 5). Therefore, in our cell assay, we showed that it is more advantageous to use a combination of 3 antisense phosphorothioate sequences than to use a combination of 2 phosphorothioate or unmodified antisense sequences plus a random sequence. These results indicate that the choice of the sequence might influence the antiviral activity of a given combination. By comparing the antiviral activity of the cocktail Srev-SDIS-SPac with that of the cocktail Srev-SDIS-SPac-RS, we also demonstrated that the number of sequences is a factor for the effectiveness of the combination. Indeed, in our assays, we could achieve higher antiviral activity with the 3-ODN combination than with the 4-ODN combination at 100 pM final concentration. This result cannot be explained only by a dilution factor because a high level of inhibition was obtained by using a combination of 3 ODNs at 10 pM final concentration (Figure 1), thus a combination made from 3.3 pM of each ODN, compared with 25 pM of each ODN for the 4-ODN combination. However, one should be cautious when comparing these data because the 2 cell assays were not exactly identical. Furthermore, when we compared the antiviral activity of the 4-ODN cocktail Srev-SDIS-SPac-RS with the 3-ODN cocktail Srev-SDIS-RS, we found that a relatively smaller difference between their antiviral activity could be observed than between that of cocktails Srev-SDIS-SPac-RS and Srev-SDIS-SPac (Figure 4). This suggests that the presence of the random

sequence could be in part responsible for the reduction in the efficacy of the 4-ODN combination.

Studies on drug-combination strategy using antisense ODNs are extremely rare, making comparisons very difficult. In another study (26) in which two 28-mer antisense ODNs, anti-*rev* and anti-*gag*, were tested in combination, the authors reported that after 4 weeks of treatment, the 2-drug regimen treatment did not result in increased inhibitory activity against HIV-1 (Ba-L) isolate in monocytes/macrophages cultures at concentrations ranging from 0.1 to 1.5 μM, or HIV-1 (IIIB) isolate in peripheral blood mononuclear cells (PBMC) cultures at concentrations ranging from 0.1 to 1 μM, compared with the single-drug regimens. In contrast, in our previous study, the 3-ODN cocktail (Srev-SDIS-SPac), added free to the culture medium, inhibited the replication of an HIV-1 clinical isolate (VR2844A) in PBMCs more effectively than each ODN used alone at final concentrations of 0.1 and 0.5 μM, 7 days postinfection (Lavigne et al., under consideration for publication). However, the apparent advantage of the combination strategy in our cell assay was observed only under certain conditions (at low concentrations and for a certain period of time). It will be interesting to explore, in another study, if a combination of ODNs delivered by the DLS system will result in an increase in its antiviral effects in this cell assay, as we observed in this study in our acute assays using cell lines. However, the difference between the previously reported results and our results with clinical isolates might be explained in part by unique properties of the isolates tested and the cell assay used (27). Also, as shown in this study, the choice and the number of sequences evaluated in combination may affect the efficiency of the combination treatment and therefore may explain, in part, the difference between these 2 studies with clinical isolates.

As the additive effect of ODN combinations was, at most, low, our data revealed the high level of sequence nonspecificity of antisense ODN in the in vitro models used. High specific activity was demonstrated when using the same antisense PS-ODNs in their free form (IC50, 10-100 nM in a short-term assay), suggesting as well their non-antisense-associated inhibitory effect (data not shown). When delivered by the DLS system, IC50 of antisense ODNs was considerably reduced (1-10 pM, up to 100 000-fold), but DLS-ODNs exhibited higher sequence nonspecific activity. We restricted the scope of this study to the level of activity of the antisense oligonucleotides because the specificity of the activity has been addressed in another contribution (Lavigne et al., submitted) in which the anti-HIV activity of the phosphorothioate antisense oligonucleotides Srev, SDIS, and SPac was compared with the activity of 3 different control sequences: 2 sense sequences, a random sequence, and a G-quartet control sequence. The sequence-specific activity of the antisense sequences varied according to the cell model, the type of control, and the dose used. In particular, this investigation illustrated the importance of the nonsequence-specific activity of phosphorothioate and to a lesser extent phosphodiester ODNs on the HIV-1 replication in *in vitro* models. The presence of a G-quartet in ODN, along with particular sequences flanking the G-quartet, may also enhance nonspecific effects (28). Furthermore, CpG motifs have been found to be highly immunostimulatory in mice (29, 30). PS-oligonucleotides containing the dinucleotide motif CpG can increase immunoglobulin secretion and expression of B-cell activation markers such as MHC class II, induce interferons, augment natural killer cell activity, and stimulate the release of several interleukins from T cells. It is possible that release of interleukins such as interferon-gamma, tumor necrosis factor-alpha, or interleukin-12 may have anti-HIV activity in the assays used in this study and, thus, contribute to the random ODNs' high activity.

CONCLUSIONS

In conclusion, our observations provide new information about the beneficial effects of a combination treatment with antisense oligonucleotides in cell cultures. Our results suggest that a combination of 3 oligonucleotides delivered by a lipidic formulation could be advantageous in certain conditions of acute infection in vitro, but the advantage was not striking. In our chronic infection assay, no beneficial effects were obtained using oligonucleotides in combination. However, we were able to inhibit viral production with a cocktail of three ODNs with the same efficiency as using each ODN individually at very low concentrations (0.1 and 1 pM) with the DLS delivery system. Additive, non-antisense-, and/or antisense-associated inhibitory effects acting at early steps of the viral replicative

cycle (between the virus entry and the proviral integration), which may include sequence-dependent inhibition and nonspecific effects, might account for the higher antiviral activity displayed by our 3-phosphorothioate antisense combination (Srev-SDIS-SPac) in the acute infection model. Despite their important non-antisense specific effect, ODNs still appear to be potential anti-HIV drugs. Our data suggest that a structure/function rather than antisense/function rationale should be taken into consideration for further development of this novel class of compounds. Therefore, studies of combinations of DNA oligonucleotides with CpG ODNs or decoy ODNs to a specific protein such as transcription factor (aptamer approach) (31) need to be done. In addition, combinations with other drugs such as nucleoside and non-nucleoside analogs or protease inhibitors might be an attractive approach for the development of new strategies against HIV infection (32). Moreover, delivery with a carrier system led to much more ODN activity and should enhance the antiviral effects of such a multidrug approach.

ACKNOWLEDGEMENTS

This work was supported by grants from the Medical Research Council of Canada. C. Lavigne benefited from postgraduate scholarships from the *Fonds pour la formation de chercheurs et l'aide à la recherche* (FCAR) from the Province of Québec.

The authors want to thank sincerely Mrs. M. Fauvel for giving access to the Laboratoire de Santé Publique du Québec's P3 facilities used for virus manipulation.

REFERENCES

1. Stein CA, Cheng Y-C. Antisense oligonucleotides as therapeutics agents: Is the bullet really magical? *Science.* 1993;261:1004-1012.

2. Zhang R, Yan J, Shahinian H, et al. Pharmacokinetics of an anti-human immunodeficiency virus antisense oligodeoxynucleotide phosphorothioate (GEM 91) in HIV-infected subjects. *Clin Pharmacol Ther.* 1995;58:44-53.

3. Yamaguchi K, Papp B, Zhang D, Ali AN, Agrawal S, Byrn RA. The multiple inhibitory mechanisms of GEM 91, a *gag* antisense phosphorothioate oligonucleotide, for human immunodeficiency virus type 1. *AIDS Res Hum Retroviruses*. 1997;13:545-554.

4. Akhtar S, JJ Rossi. Anti-HIV therapy with antisense oligonucleotides and ribozymes: Realistic approaches or expensive myths? *J Antimicrob Chemother.* 1996;38:159-165.

5. Matsukura M, Zon G, Shinozuka K, et al. Regulation of viral expression of human immunodeficiency virus *in vitro* by an antisense phosphorothioate oligodeoxynucleotide against *rev* (*art/trs*) in chronically infected cells. *Proc Natl Acad Sci U S A*. 1989;86:4244-4248.

6. Kinchington D, Galpin S, Jaroszewski JW, Ghosh K, Subasinghe C, Cohen JS. A comparison of gag, pol and rev antisense oligodeoxynucleotides as inhibitors of HIV-1. *Antivir Res.* 1992;17:53-62.

7. Lisziewicz J, Sun D, Weichold FF, et al. Antisense oligodeoxynucleotide phosphorothioate complementary to Gag mRNA blocks replication of human immunodeficiency virus type 1 in human peripheral blood cells. *Proc Natl Acad Sci U S A*. 1994;91:7942-7946.

8. Anazodo MI, Wainberg MA, Friesen AD, Wright JA. Sequence-specific inhibition of gene expression by a novel antisense oligodeoxynucleotide phosphorothioate directed against a nonregulatory region of the human immunodeficiency virus type 1 genome. *J Virol*. 1995;69:1794-1801.

9. Ratner L, Haseltine W, Patarca R, et al. Complete nucleotide sequence of the AIDS virus, HTLV-III. *Nature.* 1985;313:277-284.

10. Skripkin E, Paillart J-C, Marquet R, Ehresmann B, Ehresmann C. Identification of the primary site of the human immunodeficiency virus type 1 RNA dimerization *in vitro*. *Proc Natl Acad Sci U S A*. 1994;91:4945-4949.

11. Muriaux D, Girard P-M, Bonnet-Mathonière B, Paoletti J. Dimerization of HIV-1Lai RNA at low ionic strength. *J Biol Chem*. 1995;270:8209-8216.

12. Kim S-G, Hatta T, Tsukahara S, et al. Antiviral effect of phosphorothioate oligodeoxyribonuleotides complementary to human immunodeficiency virus. *Bioorg & Med Chem*. 1995;3:49-54.

13. McBride MS, Panganiban AT. The human immunodeficiency virus type 1 encapsidation site is a multipartite RNA element composed of functional hairpin structures. *J Virol.* 1996;70:2963-2973.

14. Lever A, Gottlinger H, Haseltine W, Sodroski J. Identification of a sequence required for efficient packaging of human immunodeficiency virus type 1 RNA into virions. *J Virol.* 1989;63:4085-4087.

15. Aldovini A, Young RA. Mutations of RNA and protein sequences involved in human immunodeficiency virus type 1 packaging result in production of noninfectious virus. *J Virol*. 1990;64:1920-1926.

16. Agrawal S, Tang JY. GEM*91—An antisense oligonucleotide phosphorothioate as a therapeutic agent for AIDS. *Antisense Res Dev.* 1992;2:261-266.

17. Agrawal S. GEM 91: Antisense oligodeoxynucleotide phosphorothioate inhibitor of HIV-1 replication. Drugs of the Future. 1995;20:344-351.

18. Lavigne C, Thierry AR. Enhanced antisense inhibition of human immunodeficiency virus type 1 in cell cultures by DLS delivery system. *Biochem Biophys Res Commun*. 1997;237:566-571.

19. Popovic M, Read-Conole E, Gallo RC. T4 positive human neoplastic cell lines susceptible to and permissive for HTLV-III. *Lancet.* 1984; 2:1472-1473.

20. Popovic M, Sarngadharan MG, Read E, Gallo RC. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science.* 1984;224:497-500.

21. Thierry AR, Lunardi-Iskandar Y, Bryant JL, Rabinovich P, Gallo RC, Mahan LC. Systemic gene therapy: Biodistribution and long-term expression of a transgene in mice. *Proc Natl Acad Sci U S A.* 1995;92:9742-9746.

22. Thierry AR, Rabinovich P, Peng B, Bryant JL, Gallo RC. Characterization of liposome-mediated gene delivery: expression,

stability and pharmacokinetics of plasmid DNA. *Gene Ther*. 1997;4:226-237.

23. Pauwels R, Balzarini J, Baba M, et al. Rapid and automated tetrazolium based colorimetric assay for the detection of anti-HIV compounds. *J Virol Methods*. 1988;20:309-321.

24. Jendis J, Strack B, Volkmann S, Böni J, Mölling K. Inhibition of replication of fresh HIV type 1 patient isolates by a polypurine tract-specific self-complementary oligodeoxynucleotide. *AIDS Res Hum Retroviruses*. 1996;12:1161-1168.

25. Zelphati O, Imbach JL, Signoret N, Zon G, Rayner B, Leserman L. Antisense oligonucleotides in solution or encapsulated in immunoliposomes inhibit replication of HIV-1 by several different mechanisms. *Nucleic Acids Res*. 1994;22:4307-4314.

26. Weichold FF, Lisziewicz J, Zeman RA, et al. Antisense phosphorothioate oligodeoxynucleotides alter HIV type 1 replication in cultured human macrophages and peripheral blood mononuclear cells. *AIDS Res Hum Retroviruses*. 1995;11:863-867.

27. Crooke MR. In vitro toxicology and pharmacokinetics of antisense oligonucleotides. *Anticancer Drug Design*. 1991;6:609-646.

28. Stein CA. Phosphorothioate antisense oligodeoxynucleotides: questions of specificity. *Trends Biotechnol*. 1996;14:147-149.

29. Tamamoto T, Yamamoto S, Kataoka T, Tokunaga T. Ability of oligonucleotides with certain palindromes to induce interferon production and augment natural killer cell activity is associated with their base length. *Antisense Res Dev*. 1994;4:119-122.

30. Krieg A, Yi AK, Matson S, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature*. 1995;374:546-549.

31. White RR, Sullenger BA, Rusconi CP. Developing aptamers into therapeutics. *J Clin Invest*. 2000;106:929-934.

32. Gareth JV, Agrawal S, Byrn RA. Synergistic inhibition of HIV-1 by an antisense oligonucleotide and nucleoside analog reverse transcriptase inhibitors. *Antivir Res*. 1998;38:63-73.